FEBS 19481 FEBS Letters 417 (1997) 405–408

Lateral self-assembly of E-cadherin directed by cooperative calcium binding

Jean-René Alattia[a], James B. Ames[b], Tudor Porumb[a], Kit I. Tong[a], Yew Meng Heng[a], Peter Ottensmeyer[a], Cyril M. Kay[c], Mitsuhiko Ikura[a,*]

[a] *Division of Molecular and Structural Biology, Ontario Cancer Institute and Department of Medical Biophysics, University of Toronto, 610 University Avenue, Toronto, Ont. M5G 2M9, Canada*
[b] *Department of Neurobiology, Stanford University School of Medicine, Stanford, CA 94305, USA*
[c] *Department of Biochemistry and the Protein Engineering Network of Centre of Excellence, University of Alberta, Edmonton, Alta. T6G 2H7, Canada*

Received 19 September 1997

Abstract We report the Ca^{2+} binding characteristics of recombinant Ecad12, a construct spanning the first two repeats of epithelial cadherin, and demonstrate the links between Ca^{2+} binding and dimer formation. Sedimentation equilibrium and dynamic light scattering experiments show that weak dimerization of Ecad12 occurs in the presence of 10 mM Ca^{2+} (K_d^P = 0.17 mM), while no appreciable dimer formation was detected in the absence of Ca^{2+}. Ca^{2+}-induced dimerization was also observed in electron microscopy images of Ecad12. We conclude from Ca^{2+} titration experiments monitored by tryptophan fluorescence and flow dialysis that dimerization does not affect the equilibrium binding constant for Ca^{2+}. However, the value of the Hill coefficient for Ca^{2+} binding increases from 1.5 to 2.4 as the protein concentration increases, showing that dimer formation largely contributes to the cooperativity in Ca^{2+} binding. Based on these observations and previous crystallographic studies, we propose that calcium acts more likely as a geometrical aligner ensuring the proper assembly of cadherin molecules, rather than a simple adhesive.

Key words: Cell adhesion; Cadherin; Calcium; Dimerization

1. Introduction

It has long been recognized that calcium is a key element for maintaining cellular adhesiveness, as shown from live tissues and cultured cells dissociating whenever exposed to a calcium-depleted environment [1,2]. Cadherins are Ca^{2+}-dependent cell adhesion molecules that play a crucial role in homophilic cell-cell association [3]. The modular architecture of cadherin molecules consists of five repeats in the extracellular part, a single transmembrane region and a single intracellular domain linked to the actin filaments of the cytoskeleton via α- and β-catenins. An N-terminal fragment composed of the first two repeats of murine E-cadherin (Ecad12) was found to crystallize as a dimer, while its 3D X-ray structure revealed that the peptide stretch linking the domains of each Ecad12 molecule binds three Ca^{2+} ions, which in turn mediate a network of interactions with the opposite monomer [4]. A previous crystal structure of a single N-terminal domain of neural cadherin was also found to consist of a dimer, but it showed a different mode of interaction with no calcium ions involved in the interface between the monomers [5]. In an effort to understand the role of calcium in cadherin self-assembly, we have investigated in the present study the characteristics of calcium binding by recombinant Ecad12 and demonstrated its link to dimer formation by solution techniques and electron microscopy.

2. Materials and methods

2.1. Sedimentation equilibrium experiments

The experiments were carried out on a Beckman Model E ultracentrifuge. Sample aliquots of 100 μl were loaded into 12-mm double-sector, charcoal-filled Epon cells equipped with sapphire windows. The experiments were performed at 20°C for approximately 48 h before equilibrium photographs were taken. An assumed value of 0.73 was used for the partial specific volume of the sample. The apparent molecular weights were calculated from the slope after fitting the Ln(y) versus r^2 data to a second-degree polynomial equation, using a least-squares technique.

2.2. Light scattering

Molecular weight determinations using light scattering techniques were performed on a Dawn F multi-angle laser light-scattering photometer (Wyatt Technology Corporation, Santa Barbara, CA) according to the methodology previously described [6]. An aliquot of sample was manually injected onto a Pharmacia Superose 12 gel filtration column and the scattering intensity and RI signal of the eluting peaks were monitored. Approximately 10-fold dilution was observed upon injection of the sample of 0.2 mM protein. The RI signal was used to calculate the protein concentration, using an assumed value of 0.185 for the sample dn/dc. For each slice across a peak, a Debye plot was produced ($R_\Theta/K \times c$ versus $\sin^2\Theta/2$), in which the intercept of the extrapolation of scattering intensities to the zero angle and zero concentration yielded the reciprocal molecular mass. The apparent average molecular mass across the whole peak was then calculated from all the individual slices.

2.3. Electron microscopy

Solutions of Ecad12 (100 μg/ml) in 10 mM Tris-HCl (pH 7.5) were adsorbed on copper grids covered with butvar B-76 support film, stained with 2% phosphotungstic acid and air dried. Images were obtained on a transmission electron microscope Zeiss EM902 equipped with an energy filter. Bright field images were recorded at a magnification of 85 000 for the apo form and 50 000 for the Ca^{2+}-bound form. Selected molecules were digitized with a 64×64 pixel resolution.

2.4. Calcium binding to Ecad12

All buffers and samples were treated with Chelex to remove trace amounts of Ca^{2+}. Flow dialysis experiments were carried out at 25°C as described previously [8]. The protein solution used was 0.17 mM dissolved in 1 ml of buffer (20 mM Tris-HCl (pH 7.5), 100 mM KCl, 2 mM DTT, 100 μM NaN_3). Non-linear regression was used to fit the data to a classical Hill model. The Ecad12 sample used for the fluorescence titration was 1.5 μM dissolved in 2 ml of buffer (0.1 KCl, 50 mM HEPES, pH 7.5, 1 mM DTT) placed in a cuvette at 25°C. The intrinsic fluorescence of the protein (excitation at 290 nm and emission at 305 nm) was measured using an Aminco SLM-8000C fluorimeter. Aliquots of a standard $CaCl_2$ solution (5 mM) were added at

*Corresponding author. Fax: (1) (416) 946 6529.
E-mail: mikura@oci.utoronto.ca

PII S0014-5793(97)01333-1

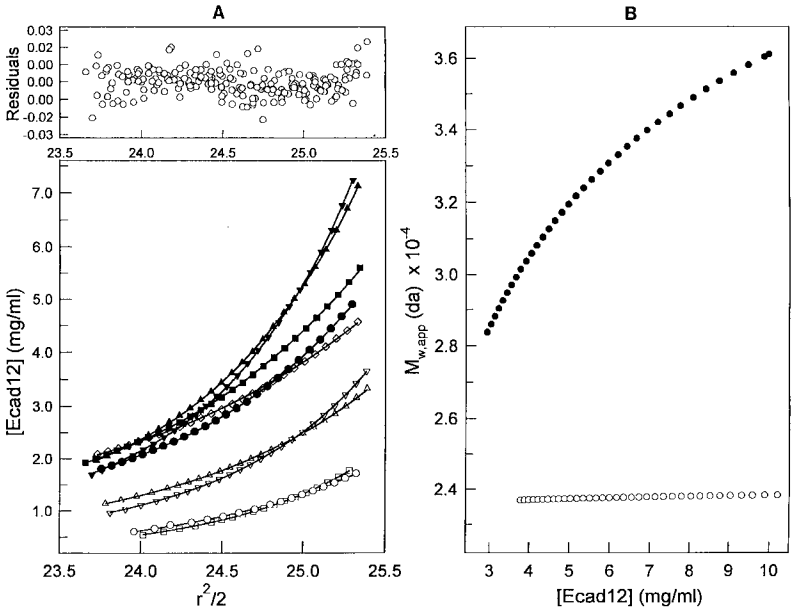

Fig. 1. Sedimentation equilibrium analysis of Ecad12. A: The results of the experiments carried out in the presence of 10 mM $CaCl_2$. A total of 9 runs were performed at different Ecad12 loading concentrations and operating speeds. The lower part of A shows the local Ecad12 concentrations as a function of the radial distance: ○ 1.05 mg/ml (14000 rpm), □ 1.05 mg/ml (16000 rpm), △ 2.11 mg/ml (13000 rpm), ▽ 2.11 mg/ml (15000 rpm), ◇ 3.24 mg/ml (11000), ● 3.24 mg/ml (13000), ■ 3.62 mg/ml (12000 rpm), ▲ 4.17 mg/ml (14000 rpm), ▼ 4.17 mg/ml (15000 rpm). The upper figure of A shows the fitting residuals of the lower sedimentation curves to a monomer-dimer equilibrium; no specific pattern of the residuals is observed, indicating a good fit of the data. B: Apparent molecular weights as a function of local Ecad12 concentrations for apo Ecad12 (○), and in the presence of 10 mM $CaCl_2$ (●).

regular intervals and the fluorescence was monitored as a function of added Ca^{2+}. The fluorescence signal was corrected for dilution and background Raman scattering. The observed fluorescence as a function of added Ca^{2+} can be expressed as fractional saturation using $Y=(F-F_{min})/(F_{max}-F_{min})$, where F denotes the observed fluorescence signal at a given Ca^{2+} concentration, and F_{min} is the fluorescence observed at very low Ca^{2+} (<100 nM). The free Ca^{2+} concentration is then calculated as $Ca_{free}=Ca_{total}-(3\times Y\times[P])$, where Ca_{total} denotes the total 'added' Ca^{2+} concentration, and [P] denotes the molar concentration of Ecad12. This expression assumes that a total of three Ca^{2+} ions bind to the protein at saturation as demonstrated by the flow dialysis experiments.

3. Results

Sedimentation equilibrium centrifugation and dynamic light scattering experiments were performed to determine the oligomeric state of Ecad12 in the presence and absence of 10 mM $CaCl_2$. The sedimentation equilibrium runs were carried out at 20°C for approximately 48 h with initial protein loading concentrations of 0.22 and 0.04–0.17 mM for the Ca^{2+}-free and the Ca^{2+}-loaded samples, respectively. The sedimentation equilibrium data in the presence of calcium show evidence of a concentration-dependent monomer-dimer equilibrium (Fig. 1A,B). The data can be fitted to a two-state model quite well, resulting in a calculated dissociation constant, K_d^P, of 0.17 mM. At the much lower concentration of 0.02 mM, the dynamic light scattering data suggest that Ecad12 is mainly monomeric, in spite of the presence of 10 mM $CaCl_2$, which is consistent with the value of K_d^P determined from sedimen-

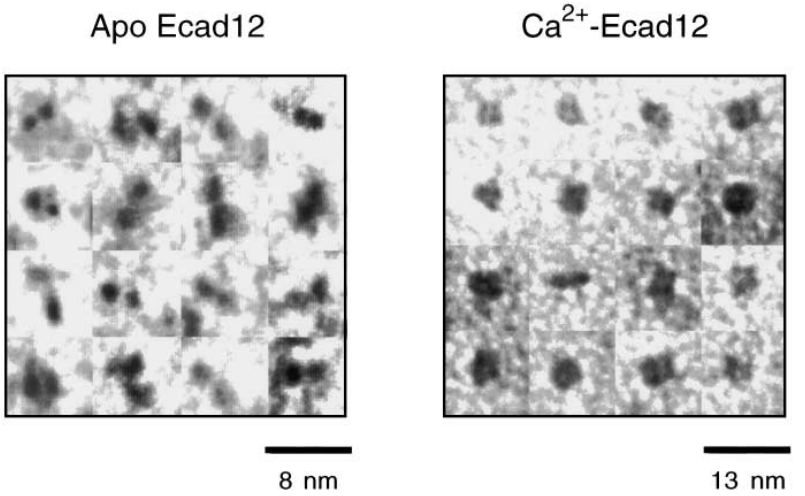

Fig. 2. Electron micrographs of negatively stained Ecad12 in the absence and presence of 10 mM $CaCl_2$.

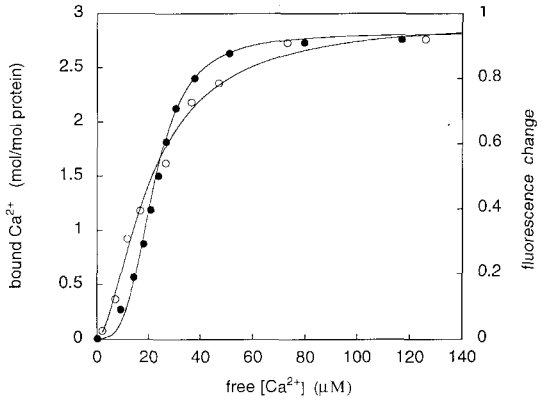

Fig. 3. Calcium binding to Ecad12. Ca^{2+} titrations were monitored by flow dialysis (●, left scale) and tryptophan emission fluorescence (○, right scale).

tation experiments. In the absence of calcium, only a single species of an average apparent molecular weight of 23767±950 Da was observed for concentrations 0.16–0.4 mM, in close agreement with the calculated monomeric molecular mass of 24698 Da. Therefore, Ca^{2+}-free Ecad12 is monomeric in solution.

We used electron microscopy to determine the shape of Ca^{2+}-free Ecad12 molecules as only the Ca^{2+}-bound form could be successfully crystallized in previous attempts. Negatively stained micrographs of Ca^{2+}-free Ecad12 showed single molecules, each of which contains two domains (Fig. 2, left). The orientation of the domains relative to each other within a molecule was variable from one molecule to another, suggesting that the linker region between the first and second domains (repeats), home to the Ca^{2+} binding sites, is relatively flexible. In contrast, addition of 10 mM $CaCl_2$ resulted in the formation of tetrad-shaped structures of dimeric Ecad12 molecules (Fig. 2, right). This observation on Ca^{2+}-loaded Ecad12 is consistent with previous crystallographic and electron microscopy studies showing that calcium extends and rigidifies the extracellular segment of E-cadherin [7] and that lateral association of adjacent extracellular segments occurs at the N-terminal tip of the molecule [8].

The crystal structure of the Ecad12 dimer, in which a pair of three Ca^{2+} ions forms intimate contacts at the dimer interface, predicts that Ca^{2+} ions would bind to the cadherin dimer in a cooperative manner [4]. To investigate in detail the nature of Ca^{2+} binding by Ecad12, we have performed continuous flow dialysis experiments [9] at peptide concentrations of 0.17 and 0.35 mM, at which dimer formation can take place. Similar results were obtained from both titrations. The shape of the calcium binding curve (Fig. 3) demonstrates a high cooperativity, confirmed by a value of 2.4 for the Hill coefficient as calculated from a non-linear fit of the data. The coefficient is close to the maximum value of 3 expected for a molecule with three binding sites. In agreement with the crystal structure of Ecad12, the number of these sites is confirmed from the ratio of bound calcium, at the end of the titration, to the total protein concentration. Interestingly, the data show that the protein sample, which contains roughly equal populations of Ecad12 in monomeric and dimeric forms, binds calcium with an apparent stoichiometry of 3 ions per polypeptide chain. An apparent dissociation constant for calcium binding, K_d^{Ca}, of 23 μM was obtained from the fit of the data to a Hill model. This value of K_d^{Ca} is similar to the value determined by Koch et al. (30 μM) [10] for the entire extracellular region of E-cadherin. These results indicate that Ecad12, which spans the two N-terminal domains of E-cadherin, retains the Ca^{2+} binding properties of the whole extracellular part.

To investigate the Ca^{2+} binding properties of monomeric Ecad12, Ca^{2+} titration at very low protein concentrations (1.5 μM) was monitored by tryptophan fluorescence spectroscopy. Most markedly, the midpoint of the titration curve (20 μM) (Fig. 3) agreed well with the value of K_d^{Ca} (23 μM) obtained at higher protein concentrations by flow dialysis. The experiment was repeated with protein concentrations ranging from 1.5 to 50 μM, for which similar K_d^{Ca} values (20±3 μM) were obtained. Therefore, the dissociation of the Ecad12 dimer does not significantly affect its binding constant for calcium. On the other hand, the calculated Hill coefficient of 1.5±0.2, a value that indicates some cooperative behavior in the binding of Ca^{2+} ions to monomeric Ecad12, is lower than the value observed under flow dialysis conditions (2.4) where dimer formation can occur. The crystal structure of the Ecad12 dimer showed two types of interactions between the Ca^{2+} binding sites [4]. First, intramolecular interactions involving side chains of residues Glu-11, Glu-69, Asp-103 and Asp-136 link the three Ca^{2+} ions bound to the Ecad12 monomer. Sec-

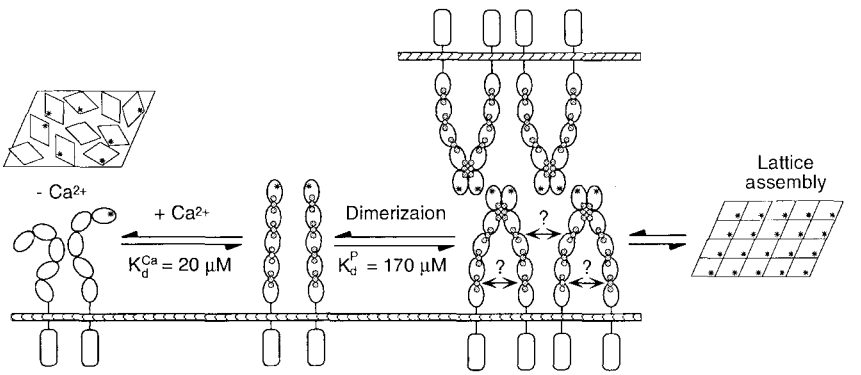

Fig. 4. Schematic representation of the proposed mechanism by which Ca^{2+}-organized cadherin assemblies are formed at the cell surface. Extracellular cadherin repeats are represented by open ovals, Ca^{2+} ions by small shaded spheres, adhesion sites by asterisks and the cytoplasmic domain by a rectangle. Only the two N-terminal repeats are shown to interact in dimers. Other repeats might be involved in contacts either within the same dimer or with other dimers. The cytoplasmic domain and/or the molecules from the opposite cell may also contribute to the cadherin lattice assembly. The disordered (−Ca^{2+}) cadherin lattice is represented at the top, where adhesion sites might be unformed or assume random orientations.

ond, residues Asp-100, Gln-101, Asn-102 and Asn-143 coordinate Ca^{2+} ions on one monomer while forming intermolecular hydrogen bonds with the opposite monomer, either directly or via water molecules. The abolition of the intermolecular interactions in dilute protein conditions can affect the network linking the Ca^{2+} binding sites, and hence the cooperative behavior of Ca^{2+} binding by Ecad12.

4. Discussion

The importance of Ca^{2+} ions in stabilizing the interactions between Ecad12 monomers has been previously reported [4,11]. Based on two major observations, the present study provides further definition of the key role played by Ca^{2+} ions in the lateral interaction between cadherin molecules. First, the association of Ca^{2+}-bound Ecad12 molecules is intrinsically weak as shown from the relatively high value of K_d^P (0.17 mM). Second, the values of K_d^{Ca} remain similar between the monomeric and dimeric forms, indicating that dimerization is not required for effective binding of three calcium ions to Ecad12. Interestingly, the cooperativity in Ca^{2+} binding is enhanced by dimer formation. Considering these and the previous studies mentioned above, we propose that one of the essential roles of Ca^{2+} ions is to ensure the proper orientation of cadherin molecules in an aligned extracellular lattice suitable for proper adhesive contacts. The crystal structure of the Ecad12 dimer showed that Ca^{2+} ions are coordinated by backbone and side chain oxygen atoms of residues that are strategically located at the interface between the first and second cadherin domains. As mentioned earlier, some of the residues that are involved in calcium coordination form hydrogen bonds with the opposite monomer, either directly or via bound water molecules. Thus, an intricate network of interacting Ca^{2+} ions, water molecules and Ecad12 side chains results in a very specific geometry that would fix the orientation between the individual monomers. Similar arrangements might occur at putative Ca^{2+} binding sites in other cadherin domains, possibly allowing extra contacts between the molecules. Grouping, as well as organizing cadherin structures, can yield the necessary strength for adhesion. Lateral clustering interactions between cadherin monomers on the surface of the plasma membrane are probably not limited to the N-terminal tip of the cadherin molecule; other extracellular parts and/or the intracellular domain are possibly involved [11,12]. This corroborates the observation showing that Ca^{2+} depletion by EGTA does not entirely disrupt cell-cell contacts in F9 teratocarcinoma cells [13].

Fig. 4 illustrates the proposed mechanism by which the Ca^{2+}-dependent assembly of cadherin molecules forms a stable cell adhesion surface on the plasma membrane. First, apocadherin monomers with flexible linkers bind Ca^{2+} ions, resulting in the overall rigidification of the molecule. Similarly important is that calcium binding restrains the positions of the individual adhesion sites to those suitable for the formation of a uniform cell-cell adhesion lattice. Ca^{2+} ions, and metal ions in general, can potentially provide the geometrical scaffolding through their coordinating valences, allowing an organized positioning of the polypeptide chains to occur. Such organized structures at low concentrations would associate loosely, as exemplified by the weakness of the Ecad12 dimer in solution. However, the key to stable adhesion would lie in the collective assembly of a number of properly oriented structures. If a critical concentration of cadherin molecules is met at the cell surface, dimer formation occurs with a geometry guided by Ca^{2+} ions. The dimerization step, together with possible grouping events originating in the cytoplasm or involving other cadherin repeats, are crucial for the formation of an organized assembly. Finally, the cadherin cluster can interact with a geometrically compatible assembly that is similarly formed at the opposite cell surface to achieve cadherin-mediated cell-cell adhesion.

The necessity for lateral dimerization of transmembrane adhesion molecules may stem from outward or inward signal transduction processes. Pairing of cytoplasmic domains, brought together by intracellular forces, is likely to facilitate the dimerization of the extracellular portions of the cadherin molecules. In turn, this might alter the organization of the adhesion surface, resulting in modulation of the homophilic binding affinity [12]. Conversely, extracellular events can transmit signals into the cytoplasm through cadherin dimers, with a subsequent structural change in the cytoplasmic plaque bound to the cytoskeleton. Interestingly, various other receptors with single transmembrane segments transmit signals by virtue of their dimer formation ability, such as protein-tyrosine kinase receptors, cytokine receptors, or receptors for the tumor necrosis factor [14–16].

Acknowledgements: We thank Leslie D. Hicks for expert technical support in light scattering and analytical ultracentrifugation, Jim Rini, Masatoshi Takeichi and Michael Overduin for stimulating discussions and critical reading of the manuscript, and Gregory J. Czarnota for providing initial electron microscopy images. The fluorescence experiments were carried in the laboratory of Lubert Stryer at Stanford University. This work was supported by grants to M.I. from the National Cancer Institute of Canada. J.-R.A. acknowledges the Ontario Cancer Institute/Amgen award of post-doctoral fellowships. J.B.A. was supported by a NIH post-doctoral fellowship (EY06418-02). M.I. is a Howard Hughes Medical Research International Research Scholar.

References

[1] Kartenbeck, J., Schmid, E., Franke, W.W. and Geiger, B. (1982) EMBO J. 6, 725–732.
[2] Sedar, A.W. and Forte, J.G. (1964) J. Cell. Biol. 22, 173–188.
[3] Takeichi, M. (1991) Science 251, 1451–1455.
[4] Nagar, B., Overduin, M., Ikura, M. and Rini, J.M. (1996) Nature 380, 360–364.
[5] Shapiro, L. et al. (1995) Nature 374, 327–336.
[6] Wyatt, P.J. (1993) Anal. Chim. Acta 272, 1–40.
[7] Pokutta, S., Herrenknecht, K., Kemler, R. and Engel, J. (1994) Eur. J. Biochem. 223, 1019–1026.
[8] Tomschy, A., Fauser, C., Landwehr, R. and Engel, J. (1996) EMBO J. 15, 3507–3514.
[9] Porumb, T. (1994) Anal. Biochem. 220, 227–237.
[10] Koch, A.W., Pokutta, S., Lustig, A. and Engel, J. (1997) Biochemistry 36, 7697–7705.
[11] Brieher, W.M., Yap, A.S. and Gumbiner, B.M. (1996) J. Cell. Biol. 135, 487–496.
[12] Yap, A.S., Brieher, W.M., Pruschy, M. and Gumbiner, B.M. (1997) Curr. Biol. 7, 308–315.
[13] Hirano, S., Nose, A., Hatta, K., Kawakami, A. and Takeichi, M. (1987) J. Cell. Biol. 105, 2501–2510.
[14] Gumbiner, B.M. (1997) Curr. Biol. 7, R443–R446.
[15] Heldin, C.H. (1995) Cell 80, 213–223.
[16] Stock, J. (1996) Curr. Biol. 6, 825–827.